recommending that all patients with HCAP be treated empirically with multiple antibiotics, directed against multidrug-resistant (MDR) pathogens [1]. Recently, however, there has been opposition to this approach, because not all HCAP patients are at risk for MDR pathogen infection [2, 3].

Several recent studies have shown that HCAP patients are a heterogeneous group. Some studies, mainly from the United States, reported a higher frequency of drug-resistant pathogens and mortality in HCAP than in CAP [4, 5], whereas several European studies found a lower frequency of drug-resistant pathogens and demonstrated the efficacy of narrow-spectrum therapy [6–8]. Nursing home–acquired pneumonia (one type of HCAP) in Japan and Spain has a mortality rate and etiology more similar to CAP than to hospital-acquired pneumonia (HAP) [9–11]. It is desirable not to use aggressive empiric combination therapy for HCAP patients if it is not necessary, as it may possibly lead to drug toxicity, as well as promoting future antimicrobial resistance [12].

In 2009 Brito and Niederman proposed a therapeutic strategy that divided HCAP patients into 4 groups based on severity of illness (ie, the need for mechanical ventilation or intensive care unit [ICU] admission) and the presence of other risk factors for MDR pathogens [2]. CAP therapy was recommended for HCAP patients with 0–1 MDR risk factor, and HAP therapy with a 2- or 3-drug regimen was recommended for HCAP patients with ≥2 MDR risk factors.

In reviewing previous reports of HCAP in Japan [10, 13], we found that not every patient with HCAP had MDR pathogens or required treatment for them. Furthermore, there was no Japanese guideline for HCAP at the time of this investigation. Therefore, we conducted a prospective multicenter cohort study to evaluate the efficacy of the above algorithm for guiding the initial empiric therapy for HCAP.

METHODS

This prospective multicenter cohort study was conducted between June 2009 and May 2011 at 6 Japanese hospitals. Informed consent was obtained from eligible patients. The study was approved by the Committee for Ethics and Clinical Investigation of each participating hospital, and was registered with the Japan Medical Association Center for Clinical Trials (JMA-IIA00054).

Definitions of CAP and HCAP and Algorithm for Therapy

All patients were hospitalized and had radiographically confirmed pneumonia and appropriate clinical findings (Supplementary Data). Patients with hospital-acquired pneumonia (HAP), pulmonary tuberculosis, and an infiltrate other than pneumonia were excluded. HCAP and CAP were defined according to the ATS/IDSA guidelines [1]. HCAP inclusion criteria are listed in the Supplementary Data.

HCAP patients were classified into 4 groups on the basis of severity of the illness and the presence of other risk factors for MDR pathogens (Figure 1). Severe illness included needing mechanical ventilation or ICU admission. Other risk factors for MDR pathogens were (1) immune suppression (Supplementary Data), (2) hospitalization within the last 90 days, (3) poor functional status (Barthel Index score <50), and (4) antibiotic therapy within the past 6 months.

HCAP patients with 0–1 MDR risk factor (groups 1 and 3) were treated with CAP therapy (a β-lactam in combination with a macrolide, or a quinolone), whereas HCAP patients with ≥2 MDR risk factors (groups 2 and 4) were treated with HAP therapy (2- or 3-drug regimen that included an antipseudomonal β-lactam in combination with a quinolone or aminoglycoside, plus either optional linezolid or vancomycin) (Figure 2).

Outcome Measures and Clinical Assessment

We compared the baseline characteristics, presence of MDR risk factors, etiology, antibiotic regimens, and outcomes for (1) CAP vs HCAP patients, (2) HCAP patients with 0–1 MDR risk factor vs patients with ≥2 MDR risk factors, and (3) nonsevere HCAP vs severe HCAP patients.

The CURB-65 (confusion, urea, respiratory rate, blood pressure, age ≥65) and Pneumonia Severity Index (PSI) scores were used to assess the severity of pneumonia [14, 15]. The activities of daily living (ADL) were evaluated using the Barthel Index, which was scored from 0 to 100 based on 10 variables (Supplementary Data) [16]. Patients with <50 points were defined as having poor functional status.

Inappropriate therapy was defined when a defined etiologic pathogen was resistant to the initial antibiotic regimen. Measured outcomes were 30-day mortality and initial treatment failure, defined as a change from the initial therapeutic agents after 48 hours, due to detection of pathogens resistant to initial therapy or clinical instability (Supplementary Data). Patients were followed from the time of onset of pneumonia for 30 days or until the day of death.

Microbiologic Evaluation

Sputum, nasopharyngeal swabs (for polymerase chain reaction [PCR] and culture), serum, urine, and 2 samples of blood were collected for microbiologic examination, and microimmunofluorescence was used to measure for immunoglobulin G and immunoglobulin M antibodies against *Chlamydophila* (Supplementary Data) [17–20]. The antimicrobial susceptibility of isolated bacterial pathogens was assessed on the basis of the minimum inhibitory concentration [21]. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and extended-spectrum β-lactamase

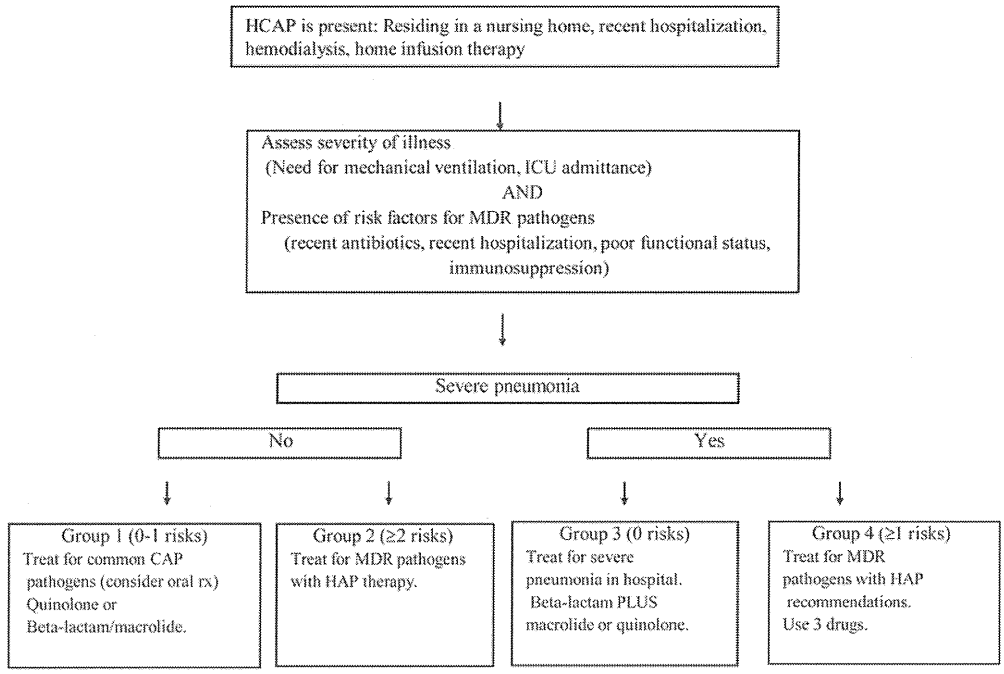

Figure 1. An algorithm for healthcare-associated pneumonia therapy based on severity of illness on admission and the presence of risk factors for multidrug-resistant pathogens. Adapted from Brito and Niederman [2]. Abbreviations: CAP, community-acquired pneumonia; HAP, hospital-acquired pneumonia; HCAP, healthcare-associated pneumonia; ICU, intensive care unit; MDR, multidrug-resistant.

(ESBL)–producing organisms were defined as MDR pathogens based on the ATS/IDSA guidelines [1].

Etiologic Diagnosis

The etiologic diagnosis was considered definitive when any of the prespecified criteria were met (Supplementary Data). A presumptive diagnosis of a pathogen was made if there was heavy growth ($\geq 10^7$ colony-forming units/mL) in culture of a predominant bacterium from sputum and Gram staining was considered consistent with that pathogen.

Statistical Analysis

Data were expressed as the mean ± standard deviation and analyzed using the SPSS software package (version 19.0; SPSS Inc, an IBM company). Univariate analysis was carried out using the χ^2 test or Fisher exact test for categorical data and the Mann-Whitney *U* test for continuous variables. Univariate and multivariate logistic regression analyses were performed to predict 30-day mortality (dependent variable). The independent variables are listed in the Supplementary Data. Variables that showed a significant difference ($P < .1$) in the univariate analysis were included in the forward likelihood ratio stepwise multivariate logistic regression model to determine if any of them were independently related to outcome. The Hosmer-Lemeshow goodness-of-fit test was performed to assess the overall fit of the model. All reported *P* values are 2-tailed, with a *P* value <.05 considered statistically significant.

RESULTS

Patient Characteristics

Four hundred sixty-nine patients with suspected pneumonia were enrolled, with 24 excluded (Supplementary Data). The remaining 445 patients included 124 with CAP and 321with HCAP, with characteristics summarized in Table 1. Among the HCAP patients, 292 had a history of contact with the hospital in the past 30 days and 145 were residents of nursing homes or extended-care facilities. The nursing home population is compared to the other HCAP patients in Supplementary Tables 1–3.

Using the algorithm, the 321 HCAP patients were classified into groups 1 (n = 110), 2 (n = 92), 3 (n = 41), and 4 (n = 78) (Figure 2). HCAP patients were significantly older than CAP patients (mean, 79.7 ± 10.4 vs 68.7 ± 15.9 years; $P < .001$) and had altered mental status more often. Patients with at least 1 underlying disease were more common with HCAP (100%) than with CAP (59.7%). HCAP patients had significantly more MDR risk factors than CAP patients (mean, 1.8 ± 1.3 vs

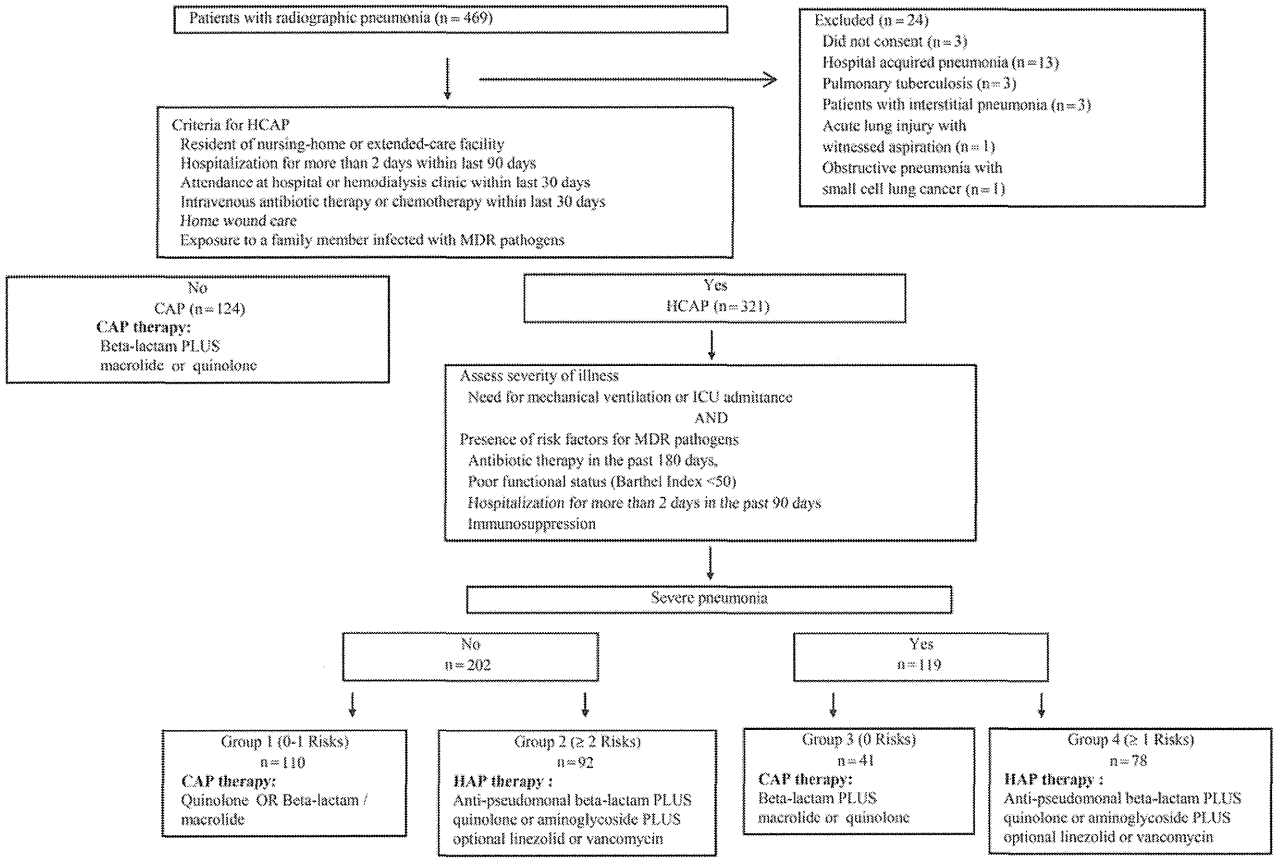

Figure 2. Classification of pneumonia patients in the current study using the algorithm in Brito and Niederman [2]. The specific antibiotic regimens for community-acquired and healthcare-associated pneumonia therapy are listed. Abbreviations: CAP, community-acquired pneumonia; HAP, hospital-acquired pneumonia; HCAP, healthcare-associated pneumonia; ICU, intensive care unit; MDR, multidrug-resistant.

0.2 ± 0.4; $P < .001$; Table 1). ADL, evaluated with the Barthel Index, and the nutritional status (serum albumin) were significantly more impaired in HCAP than in CAP patients (Table 2).

Etiology of Pneumonia

A microbiologic diagnosis was established in 51.6% (64/124) of CAP patients and 66% (212/321) of HCAP patients. The diagnosis was definitive in 67.2% (43/64) and presumptive in 32.8% (21/64) of the CAP patients, and in 67.5% (142/212) and 33% (70/212) of the HCAP patients, respectively. Diagnostic procedures used to define etiology are shown in Table 3. A mixed population of pathogens was detected in 12.9% (16/124) of CAP patients and 19.3% (62/321) of HCAP patients.

Table 4 shows the frequency of the etiologic microorganisms in each patient group. *Streptococcus pneumoniae* was the most frequent pathogen in both HCAP and CAP patients. The frequencies of *S. aureus* (11.5% vs 0.8%; $P < .001$), MRSA (6.9% vs 0%; $P = .003$), Enterobacteriaceae (7.8% vs 2.4%; $P = .037$), *P. aeruginosa* (6.9% vs 0.8%; $P = .01$), and MDR pathogens were significantly higher in HCAP than in CAP patients (15.3% vs 0.8%; $P < .001$). *Chlamydophila pneumoniae* was equally frequent in both CAP (4.1%) and HCAP (5%) patients, whereas *Mycoplasma pneumoniae* was more common in CAP than in HCAP patients (8.9% vs 4.0%; $P = .044$; Table 4). Among the HCAP patients with ≥2 MDR risk factors (groups 2 and 4), compared to those with 0–1 risk factor (groups 1 and 3), the frequencies of *S. aureus* (17.6% vs 4.6%; $P < .001$), MRSA (12.9% vs 0%; $P < .001$), *P. aeruginosa* (11.2% vs 2%; $P = .001$), and MDR pathogens (27.1% vs 2%; $P < .001$) were higher (Table 5).

Severity and Prognosis

The 30-day mortality was higher in HCAP patients than in CAP patients (13.7% vs 5.6%; $P = .017$), consistent with a higher severity of illness in the HCAP patients, as reflected by higher ($P < .001$) values for the CURB-65 score and the PSI (Table 2). Among HCAP patients, the 30-day mortality was significantly lower in patients with 0–1 risk factor (groups 1 and 3) compared to patients with ≥2 MDR risk factor (groups 2 and 4) (8.6% vs 18.2%; $P = .012$; Figure 3). Similarly, the mean PSI score, CURB-65 score, and need for ICU admission and mechanical ventilation were significantly lower in HCAP

Table 1. Patient Characteristics (N = 445)

Characteristic	CAP (n = 124)	All HCAP (n = 321)	Group 1 in HCAP (n = 110)	Group 2 in HCAP (n = 92)	Group 3 in HCAP (n = 41)	Group 4 in HCAP (n = 78)	*P* Value[a]
Age, y, mean ± SD	68.7 ± 15.9	79.7 ± 10.4	78 ± 10.7	80.5 ± 10.6	82 ± 10.4	80.1 ± 9.7	<.001
Age range, y	16–94	28–104	28–98	29–96	38–98	49–104	. . .
Sex, male	78 (62.9)	203 (63.2)	66 (60)	56 (60.9)	29 (70.7)	52 (66.7)	.947
Smoking history	39 (31.5)	149 (46.4)	60 (54.5)	36 (39.1)	20 (48.8)	33 (42.3)	.004
Alcohol history	27 (21.8)	53 (16.5)	22 (20)	10 (10.9)	9 (22)	12 (15.4)	.195
Bedridden	2 (1.6)	102 (31.8)	16 (14.5)	38 (41.3)	0	48 (61.5)	<.001
Attendance at hospital or hemodialysis clinic within last 30 d	0	292 (91)	99 (90)	88 (95.7)	29 (70.7)	76 (97.4)	. . .
Resident of nursing home or extended-care facility	0	145 (45.2)	27 (24.5)	54 (58.7)	11 (26.8)	53 (67.9)	. . .
Hospitalization for >2 d during last 90 d	0	111 (34.6)	3 (2.7)	66 (71.7)	0	32 (41)	. . .
Intravenous antibiotic therapy or chemotherapy within last 30 d	0	62 (19.3)	11 (10)	34 (37)	1 (2.4)	16 (20.5)	. . .
Exposure to a family member infected with MDR pathogen	0	20 (6.2)	7 (6.4)	7 (7.6)	0	6 (7.7)	. . .
Home wound care	0	6 (1.9)	1 (0.9)	2 (2.2)	0	3 (3.8)	. . .
No. of risk factors for drug-resistant pathogens, mean ± SD	0.2 ± 0.42	1.8 ± 1.3	0.58 ± 0.52	2.53 ± 0.7	1 ± 0	3 ± 1.2	<.001
Need for mechanical ventilation or ICU admittance	10 (8.1)	119 (37.1)	0	0	41 (100)	78 (100)	. . .
Antibiotic therapy in the past 6 mo	4 (3.2)	101 (31.5)	7 (6.4)	73 (79.3)	0	31 (39.7)	. . .
Hospitalization for >2 d during last 90 d	0	111 (34.6)	4 (3.6)	71 (77.2)	0	36 (46.2)	. . .
Poor functional status (Barthel Index score <50)	15 (12.1)	184 (57.3)	37 (33.6)	74 (80.4)	0	69 (88.5)	. . .
Immunosuppression	2 (1.6)	46 (14.3)	7 (6.4)	20 (21.7)	0	19 (24.4)	. . .
Influenza vaccination within 1 y	62 (50)	220 (68.5)	69 (62.7)	70 (76.1)	23 (56.1)	58 (74.4)	<.001
Pneumococcal vaccination within 5 y	4 (3.2)	26 (8.1)	8 (7.3)	12 (13)	1 (2.4)	5 (6.4)	.066
Comorbidity							
Cerebrovascular disease	17 (13.7)	111 (34.6)	26 (23.6)	34 (37)	13 (31.7)	38 (48.7)	<.001
Chronic pulmonary disease	34 (27.4)	73 (22.7)	31 (28.2)	17 (18.5)	10 (24.4)	15 (19.2)	.301
Diabetes mellitus	14 (11.3)	41 (12.8)	9 (8.2)	15 (16.3)	6 (14.6)	11 (14.1)	.67
Congestive heart failure	3 (2.4)	25 (7.8)	10 (9.1)	6 (6.5)	4 (9.8)	5 (6.4)	.037
Other heart disease	6 (4.8)	31 (9.7)	9 (8.2)	8 (8.7)	4 (9.8)	10 (12.8)	.099
Malignancy	1 (0.8)	47 (14.6)	20 (18.2)	12 (13)	4 (9.8)	11 (14.1)	<.001
Psychological disorder	2 (1.6)	18 (5.6)	5 (4.5)	2 (2.2)	4 (9.8)	7 (9)	.068
Chronic liver disease	3 (2.4)	14 (4.4)	5 (4.5)	5 (5.4)	2 (4.9)	2 (2.6)	.338
Chronic renal disease	5 (4)	16 (5)	2 (1.8)	4 (4.3)	4 (9.8)	6 (7.7)	.806
Neurological disease	2 (1.6)	15 (4.7)	3 (2.7)	6 (6.5)	2 (4.9)	4 (5.1)	.103
Collagen vascular disease	3 (2.4)	7 (2.2)	2 (1.8)	3 (3.3)	0	2 (2.6)	.879
Gastrostomy	1 (1.8)	23 (7.2)	1 (0.9)	15 (16.3)	0	7 (9)	.008
Postsurgery	7 (5.6)	17 (5.3)	5 (4.5)	3 (3.3)	1 (2.4)	4 (5.1)	.884
Other	3 (2.4)	13 (4)	3 (2.7)	10 (10.9)	0	0	.408
None	50 (40.3)	0	0	0	0	0	<.001

All data are presented as No. (%) unless otherwise specified.

Abbreviations: CAP, community-acquired pneumonia; HCAP, healthcare-associated pneumonia; ICU, intensive care unit; MDR, multidrug resistant; SD, standard deviation.

[a] Compared with CAP and all HCAP.

patients with 0–1 risk factor than in the patients with ≥2 MDR risk factors: 113.8 ± 33.4 vs 127.5 ± 31.3 ($P < .001$), 2.1 ± 1.1 vs 2.5 ± 1.2 ($P = .002$), and 27.2% vs 45.9% ($P < .001$), respectively.

HCAP patients with and without severe illness were compared (groups 3 and 4 vs groups 1 and 2). The 30-day mortality rate for those with severe illness was higher (30.3%) than for

Table 2. Activities of Daily Living, Severity, and Outcome (N = 445)

Outcome Measure	CAP (n = 124)	All HCAP (n = 321)	Group 1 in HCAP (n = 110)	Group 2 in HCAP (n = 92)	Group 3 in HCAP (n = 41)	Group 4 in HCAP (n = 78)	*P* Value[a]
Barthel Index score (0–100)	85.8 ± 21	48.6 ± 48.3	70.1 ± 38.3	32.6 ± 37.7	85.5 ± 13.3	18 ± 30	<.001
Albumin, g/dL	3.6 ± 0.6	3.2 ± 0.6	3.5 ± 0.5	3.1 ± 0.7	3.4 ± 0.5	2.9 ± 0.6	<.001
Blood urea nitrogen, mg/dL	19.6 ± 16.3	26.3 ± 20.4	21.8 ± 14.8	23.8 ± 16.4	31.4 ± 17	31.6 ± 27.7	<.001
C-reactive protein, mg/dL	12.4 ± 7.7	11.1 ± 7.4	11.4 ± 7.7	10.6 ± 7.1	10.8 ± 8.8	11.2 ± 7.3	.089
Procalcitonin, ng/mL	1.1 ± 6	3.1 ± 11.7	2.4 ± 7.1	3.2 ± 13.4	2.4 ± 4.3	4.3 ± 15.3	<.001
PaO_2/FiO_2, mm Hg	317.8 ± 90.1	265.9 ± 91.6	308.1 ± 68.2	290 ± 76	207.7 ± 91.7	208.1 ± 93.5	<.001
PSI score	90.1 ± 28	121.1 ± 33.0	101.1 ± 22.8	113.2 ± 25.5	148.2 ± 35.2	144.1 ± 29.6	<.001
Mild (I–III)	78 (62.9)	54 (16.8)	38 (34.5)	11 (12)	2 (4.9)	3 (3.8)	. . .
Moderate (IV)	36 (29.0)	162 (50.5)	60 (54.5)	67 (72.8)	12 (29.3)	23 (29.5)	. . .
Severe (V)	10 (8.1)	105 (32.7)	12 (10.9)	14 (15.2)	27 (65.9)	52 (66.7)	<.001
CURB-65 score (0–5)	1.2 ± 1.0	2.3 ± 1.1	1.7 ± 0.9	2.0 ± 0.9	3.0 ± 0.8	3.2 ± 1.1	<.001
Mild (0–1)	84 (67.7)	79 (24.6)	46 (41.8)	28 (30.4)	0	5 (6.4)	. . .
Moderate (2)	22 (17.7)	86 (26.8)	23 (20.9)	40 (43.5)	10 (24.4)	13 (16.7)	. . .
Severe (3–5)	18 (14.5)	136 (42.4)	21 (19.1)	24 (26.1)	31 (75.6)	60 (76.9)	<.001
Initial treatment failure	14 (11.3)	63 (19.6)	8 (7.3)	11 (12)	9 (22)	35 (44.9)	<.001
30-day mortality	7 (5.6)	44 (13.7)	5 (4.4)	3 (3.3)	8 (19.5)	28 (35.9)	.017

All data are presented as No. (%) or mean ± SD.

Abbreviations: CAP, community-acquired pneumonia; CURB-65, confusion, urea, respiratory rate, blood pressure, age ≥65 years; FiO_2, fraction of inspired oxygen; HCAP, healthcare- associated pneumonia; PaO_2, partial pressure of arterial oxygen; PSI, Pneumonia Severity Index; SD, standard deviation.

[a] Compared with CAP and all HCAP.

those without severe illness (4%; $P < .001$; Supplementary Table). Those with severe illness also had more impairment of ADL, as reflected by a significantly lower Barthel Index score ($P = .006$).

Therapy

Four hundred fifteen patients (93.3%) received recommended therapy. One hundred sixteen CAP patients (93.5%) received CAP therapy and 299 HCAP patients (93.1%) were treated in accordance with the algorithm [11]. The remaining 30 patients were treated with other regimens and survived, and were included in the statistical analyses. According to the algorithm, among the 321 HCAP patients, 151 could receive CAP therapy and did not need a multidrug HAP regimen (Figure 2).

A pathogen was identified in 64 CAP patients, and only 2 (3.1%) received inappropriate therapy, whereas a pathogen was identified in 212 HCAP patients, and only 15 (7.1%) received inappropriate therapy. Among low-risk HCAP patients (groups 1 and 3), the initial empiric therapy was not appropriate in 3 patients who had *P. aeruginosa*. In the 12 HCAP patients in groups 2 and 4 who received inappropriate therapy, the reason was usually because of MRSA not being empirically treated, or the presence of MDR *P. aeruginosa* or ESBL-producing gram-negative bacteria that were resistant to initial broad-spectrum therapy. HCAP patients with 0–1 MDR risk factor received inappropriate therapy using a CAP regimen 3.2% of the time (the same as for CAP patients receiving a CAP regimen), whereas those with ≥2 MDR risk factors received inappropriate therapy using a HAP regimen 10.1% of the time. In both CAP and HCAP groups, treatment failure (in 14 CAP patients [11.3%] and 63 HCAP patients [19.3%]) was more common than inappropriate therapy.

Multivariate Mortality Analysis

The Barthel Index score, partial pressure of arterial oxygen/fraction of inspired oxygen (PaO_2/FiO_2) ratio, dehydration (blood urea nitrogen), detection of MDR pathogens, nutritional status (albumin), PSI score, and initial treatment failure were significant predictors of 30-day mortality (dependent variable) in the univariate logistic regression analysis (Table 6). In the multivariate analysis, the independent predictors of 30-day mortality were the nutritional status (albumin), PSI score, and initial treatment failure. Initial treatment failure was associated with an odds ratio of mortality of 72 (Table 6). Appropriateness of initial empiric therapy did not emerge as a mortality risk in either the univariate or multivariate analysis, but the frequency of inappropriate therapy was low in both the HCAP and CAP populations (7.1% and 3.1%, respectively).

Table 3. Methods of Investigation for Causative Microorganisms

Microorganism	All Patients (N = 445)	Sputum (n = 423)	Blood (n = 427)	Pleural Effusion (n = 12)	Urinary Antigen (n = 431)	Nasopharyngeal Swab (n = 414)	Serology (n = 438)	BAL (n = 21)
Streptococcus pneumoniae	137	82	4		118			3
Staphylococcus aureus	38	36	1					2
Enterobacteriaceae	28	26	5					2
Pseudomonas aeruginosa	23	21	1	2				2
Haemophilus influenzae	19	18						2
Moraxella catarrhalis	4	4						
Streptococcus milleri group	5		1	5				
Streptococcus species	12	12	3					
Acinetobacter baumannii	2	2	1					
Anaerobes	2		2	1				
Staphylococcus epidermidis	1		1					
Chlamydophila pneumoniae	21						21	
Chlamydophila psittaci	3						3	
Mycoplasma pneumoniae	24	1				1	22	
Legionella pneumophila	1				1			
Influenza virus	15					7	8	
Respiratory syncytial virus	6						6	
Parainfluenza virus 3	7						7	

Abbreviation: BAL, bronchoalveolar lavage.

DISCUSSION

In this prospective study of 445 pneumonia patients admitted to 6 Japanese hospitals, HCAP was more common than CAP (321 patients vs 124 patients) and was associated with a higher mortality rate. HCAP patients were older, presented more often with altered mental status, and had more comorbid illness, higher disease severity, more functional impairment, and more MDR pathogens than CAP patients. However, the subgroups of HCAP patients with 0–1 MDR risk factor had a low frequency of MDR pathogens, 2%, which was similar to the 0.8% rate in CAP patients, and in contrast to the 27.1% frequency in HCAP patients with ≥2 MDR risk factors.

We used the presence of MDR risk factors to guide initial antibiotic therapy according to an algorithm (Figures 1 and 2), which was followed in 299 (93.1%) of the 321 HCAP patients. With the algorithm, CAP therapy was recommended for 151 HCAP patients with 0–1 MDR risk factor, whereas broad-spectrum HAP therapy was recommended for 170 HCAP patients with ≥2 risk factors. Using this approach, only 7.1% of HCAP patients received inappropriate therapy (3.2% in those with 0–1 risk factor and 10.1% in those with ≥2 risk factors). Despite the high rate of appropriate therapy, the mortality rate for those with HCAP was higher than for those with CAP, and the mortality rate for HCAP patients with ≥2 risk factors for MDR pathogens was higher than for those at low risk (18.2% vs 8.6%; $P = .012$; Figure 3). Inappropriate therapy was not a risk for 30-day mortality by either univariate or multivariate analysis, but in the multivariate model (Table 6), HCAP mortality risks were malnutrition (low serum albumin), high PSI score, and initial treatment failure; only in the univariate analysis was functional impairment, defined by the Barthel Index, a risk factor. Thus, patient factors, but not antibiotic choice, were associated with HCAP mortality, likely because of the high rate of appropriate therapy that occurred by following the algorithm.

Our findings are consistent with previous studies that report a higher mortality rate in patients with HCAP than with CAP [4, 5, 8]. In previous HCAP studies, the impact of therapy choice on mortality has been variable, with a few investigators reporting reduced mortality if therapy was consistent with international guidelines [7, 22]. In contrast, one study has reported that the use of a broad-spectrum HAP regimen for patients with HCAP was a risk factor for increased mortality [23], whereas in other studies, the majority of HCAP patients

Table 4. Causative Microorganisms

Microorganism[a]	CAP (n = 124)	All HCAP (n = 321)	Group 1 in HCAP (n = 110)	Group 2 in HCAP (n = 92)	Group 3 in HCAP (n = 41)	Group 4 in HCAP (n = 78)	*P* Value[b]
Streptococcus pneumoniae	31 (25)	106 (33)	42 (38.2)	24 (26.1)	17 (41.5)	23 (29.5)	.1
Staphylococcus aureus	1 (0.8)	37 (11.5)	4 (3.6)	14 (15.2)	3 (7.3)	16 (20.5)	<.001
MRSA	0	22 (6.9)	0	11 (10)	0	11 (14.1)	.003
Enterobacteriaceae[c]	3 (2.4)	25 (7.8)	4 (3.6)	10 (10.9)	0	11 (14.1)	.037
Pseudomonas aeruginosa	1 (0.8)	22 (6.9)	2 (1.8)	7 (7.6)	1 (2.4)	12 (15.4)	.01
Haemophilus influenzae	8 (6.5)	11 (3.4)	3 (2.7)	5 (5.4)	3 (7.3)	1 (1.3)	.157
Moraxella catarrhalis	0	4 (1.2)	2 (1.8)	0	2 (4.9)	0	.269
Streptococcus milleri group	1 (0.8)	4 (1.2)	3 (2.7)	1 (1.1)	0	0	.572
Streptococcus species	1 (0.8)	11 (3.4)	3 (2.7)	4 (4.3)	1 (2.4)	3 (3.8)	.109
Acinetobacter baumannii	0	2 (0.6)	0	0	0	2 (2.6)	.52
Anaerobes	2 (1.6)	0	0	0	0	0	.077
Staphylococcus epidermidis	0	1 (0.3)	1 (0.9)	0	0	0	.721
MDR pathogens[d]	1 (0.8)	49 (15.3)	2 (1.8)	18 (19.6)	1 (2.4)	28 (35.9)	<.001
Chlamydophila pneumoniae	5 (4.1)	16 (5)	4 (3.6)	8 (8.7)	0	4 (5.1)	.671
Chlamydophila psittaci	0	3 (0.9)	0	3 (3.3)	0	0	.374
Mycoplasma pneumoniae	11 (8.9)	13 (4.0)	6 (5.5)	2 (2.2)	2 (4.9)	3 (3.8)	.044
Legionella pneumophila	1 (0.8)	0	0	0	0	0	.279
Influenza virus[e]	6 (4.8)	9 (2.8)	6 (5.5)	0	2 (4.9)	1 (1.3)	.215
Respiratory syncytial virus	2 (1.6)	4 (1.2)	0	3 (3.3)	0	1 (1.3)	.534
Parainfluenza virus 3	3 (2.4)	4 (1.2)	1 (0.9)	1 (1.1)	0	2 (2.6)	.304
Unknown	60 (48.4)	109 (34)	44 (40)	29 (31.5)	14 (34.1)	22 (28.2)	.005

All data are presented as No. (%) unless otherwise specified (N = 445).

Abbreviations: CAP, community-acquired pneumonia; HCAP, healthcare-associated pneumonia; MDR, multidrug-resistant; MRSA, methicillin-resistant *Staphylococcus aureus*.

[a] Numbers include mixed population of pathogens (16 in CAP and 63 in All HCAP).

[b] Compared with CAP and all HCAP.

[c] Enterobacteriaceae included *Klebsiella pneumoniae* (2) and *Escherichia coli* (1) in CAP and *K. pneumoniae* (12), *E. coli* (7), *Serratia marcescens* (1), *Morganella morganii* (3), and *Proteus mirabilis* (2) in All HCAP.

[d] MDR pathogens included *Pseudomonas aeruginosa* (1) in CAP; and MRSA (22), extended-spectrum β-lactamase–producing Enterobacteriaceae (7; includes *E. coli* [4], *K. pneumoniae* [2], and *P. mirabilis* [1]), *P. aeruginosa* (22), and *Acinetobacter baumannii* (2) in All HCAP.

[e] Influenza virus included influenza A (3) and influenza B (3) in CAP and influenza A (9) in All HCAP.

were not treated with a broad-spectrum HAP regimen, but still achieved a good outcome [6, 8, 24–26]. However, in contrast to our findings, not all of these studies included patients needing ICU admission, few patients had an established etiologic diagnosis, and most patients did not have multiple MDR risk factors. In our study, we included a large number of severely ill HCAP patients needing mechanical ventilation or ICU admission (119/321 [37%]) and identified an etiologic pathogen in 66% of HCAP patients. Recently, Micek et al reported MRSA in 26.2% of HCAP patients and *P. aeruginosa* in 23%, leading them to recommend targeting of these pathogens in all of their HCAP patients, a recommendation that is not supported in our patient population [27].

We did extensive diagnostic testing to obtain a microbiologic diagnosis (Tables 3 and 4). Few epidemiological studies of HCAP have investigated atypical pathogens [28], so we routinely performed urinary antigen tests, serological tests, real-time PCR, and culture to detect atypical pathogens, and *C. pneumoniae* was diagnosed based on recommendations of the Centers for Disease Control and Prevention [29]. We found that atypical pathogens were important in both HCAP and CAP, although the incidence of *Legionella pneumophila* pneumonia is low in Japan [30, 31].

Table 5. Causative Microorganisms in Each Healthcare-Associated Pneumonia Group Classified by Risk of Multidrug Resistance

Microorganism[a]	HCAP With 0–1 MDR Risk Factor (n = 151)	HCAP With ≥2 MDR Risk Factors (n = 170)	*P* Value
Streptococcus pneumoniae	59 (39.1)	47 (27.6)	.03
Staphylococcus aureus	7 (4.6)	30 (17.6)	<.001
MRSA	0	22 (12.9)	<.001
Enterobacteriaceae[b]	4 (2.6)	21 (12.4)	.001
Pseudomonas aeruginosa	3 (2)	19 (11.2)	.001
Haemophilus influenzae	6 (4.0)	6 (3.5)	.834
Moraxella catarrhalis	4 (2.6)	0	.048
Streptococcus milleri group	3 (2.0)	1 (0.6)	.268
Streptococcus species	4 (2.6)	7 (4.1)	.47
Acinetobacter baumannii	0	2 (1.2)	.28
Staphylococcus epidermidis	1 (0.7)	0	.47
MDR pathogens[c]	3 (2)	46 (27.1)	<.001
Chlamydophila pneumoniae	4 (2.6)	12 (7.1)	.072
Chlamydophila psittaci	0	2 (1.2)	.5
Mycoplasma pneumoniae	8 (5.3)	5 (2.9)	.285
Legionella pneumophila	0	0	. . .
Influenza virus[d]	8 (5.3)	1 (0.6)	.012
Respiratory syncytial virus	0	4 (2.4)	.077
Parainfluenza virus 3	1 (0.7)	3 (1.8)	.357
Unknown	58 (38.4)	51 (30)	.112

All data are presented as No. (%) unless otherwise specified.

Abbreviations: HCAP, healthcare-associated pneumonia; MDR, multidrug-resistant; MRSA, methicillin-resistant *Staphylococcus aureus*.

[a] Numbers include mixed population of pathogens: 21 in HCAP with 0–1 MDR risk factor (groups 1 and 3) and 42 in HCAP with ≥2 MDR risk factors (groups 2 and 4).

[b] Enterobacteriaceae included *Klebsiella pneumoniae* (4) in HCAP with 0–1 risk factor and *K. pneumoniae* (8), *Escherichia coli* (7), *Serratia marcescens* (1), *Morganella morganii* (3), and *Proteus mirabilis* (2) in HCAP with ≥2 MDR risk factors.

[c] MDR pathogens included *Pseudomonas aeruginosa* (3) in HCAP with 0–1 MDR risk factor; and MRSA (22), extended-spectrum β-lactamase–producing Enterobacteriaceae (7; includes *E. coli* [4], *K. pneumoniae* [2], and *P. mirabilis* [1]), *P. aeruginosa* (19), and *Acinetobacter baumannii* (2) in HCAP with ≥2 MDR risk factors.

[d] Influenza virus included influenza A (8) in HCAP with 0–1 MDR risk factor and influenza A (1) in HCAP with ≥2 MDR risk factors.

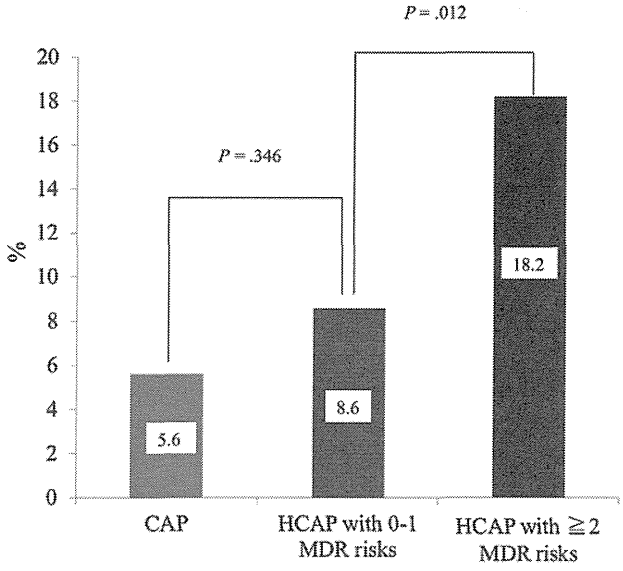

Figure 3. Thirty-day mortality in community-acquired pneumonia (CAP) and healthcare-associated pneumonia (HCAP), with each HCAP stratified by the number of multidrug-resistant (MDR) pathogen risk factors. The 30-day mortality was significantly increased in HCAP patients with ≥2 MDR risk factors compared to HCAP patients with 0–1 MDR risk factor, which in turn was not different from the mortality of CAP patients. CAP patients and HCAP patients with 0–1 MDR risk factor were treated with CAP therapy (a β-lactam and a macrolide, or a quinolone), whereas HCAP patients with ≥2 MDR risk factors were treated with hospital-acquired pneumonia therapy (2- or 3-drug regimen: an antipseudomonal β-lactam in combination with a quinolone or aminoglycoside, plus either linezolid or vancomycin if there was concern about methicillin-resistant *Staphylococcus aureus*).

Severity of illness was an important risk factor, which was associated with an increased mortality and more impaired functional status, as in other studies [32, 33]. The risk factors that we studied are similar to ones used by others, but we did not attempt to weight these risk factors by giving greater importance to specific findings, as was done by Shorr et al [34, 35]. However, their risk scoring system has not been used prospectively to guide antibiotic selection, as was done in our study.

In our study, initial treatment failure was the most important prognostic factor for 30-day mortality for HCAP patients. As this occurred in the setting of a high rate of appropriate therapy, and because the frequency of treatment failure (19.6%) exceeded the rate of inappropriate therapy (7.1%), it is likely that treatment failure reflected patient factors and was not a consequence of therapy choice. Similarly, the higher mortality in HCAP compared to CAP is more a reflection of differences in patient features, and not therapy choice.

It is important to treat HCAP patients with appropriate therapy, but without the overuse of antibiotics. The existing ATS/IDSA guidelines advocate broad-spectrum empiric therapy for all HCAP patients, whereas our data, and that of others, show that this is not necessary for all HCAP patients. Our findings emerged from a prospective, multicenter study, and all of

Table 6. Analysis of Prognostic Factors for 30-Day Mortality by Univariate and Multivariate Analysis in Healthcare-Associated Pneumonia[a]

	Univariate Analysis			Multivariate Analysis		
Prognostic Factor	OR	(95% CI)	*P* Value	OR	(95% CI)	*P* Value
Barthel Index	0.989	(.981–.997)	.007			
PaO_2/FiO_2, mm Hg	0.991	(.987–.995)	<.001			
Blood urea nitrogen, mg/dL	1.024	(1.01–1.039)	.001			
Detection of MDR pathogens	2.807	(1.344–5.862)	.006			
Albumin, g/dL	0.277	(.162–.473)	<.001	0.339	(.134–.855)	.022
PSI score	1.036	(1.025–1.048)	<.001	1.027	(1.010–1.044)	.001
Initial treatment failure	63.8	(24.589–165.744)	<.001	72.078	(21.952–236.666)	<.001

Abbreviations: CI, confidence interval; FiO_2, fraction of inspired oxygen; MDR, multidrug-resistant; OR, odds ratio; PaO_2, partial pressure of arterial oxygen; PSI, Pneumonia Severity Index.

[a] Includes 321 patients (44 died, 277 survived).

the centers were in Japan; therefore, the relevance to other countries needs to be determined, although the risk factors that we studied are universal. However, in our study the frequency of MRSA was 0% in CAP and 6.9% in HCAP, and countries with higher rates may need to modify the approach that is used. In addition, although our algorithm was generally effective, it still needs refinement, with consideration of other MDR risk factors, and with weighting of the relative importance of the risk factors that we did include. This might allow identification of a population with an even lower risk for MDR pathogens than our low-risk group, which still had these organisms present in 2% of patients. In the future, large-scale randomized controlled trials of HCAP therapy using therapeutic algorithms that consider local microbiology are needed. In addition, any algorithm will also need to consider the frequency of atypical pathogens, which may be present in both HCAP and CAP patients.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Original article

The relationship between biofilm formations and capsule in *Haemophilus influenzae*☆

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ABSTRACT

To evaluate the biofilm formation of non-typeable *Haemophilus influenzae* (NTHi) and *H. influenzae* type b (Hib) clinical isolates, we conducted the following study. Serotyping and polymerase chain reaction were performed to identify β-lactamase-negative ampicillin (ABPC)-susceptible (BLNAS), β-lactamase-negative ABPC-resistant (BLNAR), TEM-1 type β-lactamase-producing ABPC-resistant (BLPAR)-NTHi, and Hib. Biofilm formation was investigated by microtiter biofilm assay, as well as visually observation with a scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) in a continuous-flow chamber. As a result, totally 99 strains were investigated, and were classified into 4 groups which were 26 gBLNAS, 22 gBLNAR, 28 gBLPAR-NTHi and 23 Hib strains. The mean OD_{600} in the microtiter biofilm assay of gBLNAS, gBLNAR, gBLPAR-NTHi, and Hib strains were 0.57, 0.50, 0.34, and 0.08, respectively. NTHi strains were similar in terms of biofilm formations, which were observed by SEM and CLSM. Five Hib strains with the alternated type b *cap* loci showed significantly increased biofilm production than the other Hib strains. In conclusion, gBLNAS, gBLNAR, and gBLPAR-NTHi strains were more capable to produce biofilms compared to Hib strains. Our data suggested that resistant status may not be a key factor but capsule seemed to play an important role in *H. influenzae* biofilm formation.

1. Introduction

Haemophilus influenzae is a pleomorphic gram-negative rod-shaped bacterium that colonizes the human nasopharynx. Non-typeable *H. influenzae* (NTHi) can cause a variety of infections, including otitis media, sinusitis, conjunctivitis, bronchitis, and pneumonia [1], whereas *H. influenzae* type b (Hib) is reported as a common cause of meningitis and other invasive infections especially in children [2]. As well known, biofilms are defined as communities of microorganisms that attach to a surface and are enveloped in a hydrated polymeric matrix of their own synthesis [3]. It has recently been reported that NTHi is able to form biofilms in vitro [4] and on middle-ear mucosa in the chinchilla model of otitis media [5]. However, there is still very few information about biofilm formations in Hib at present. Furthermore, it has also recently been reported that β-lactamase-negative ABPC-resistant (BLNAR) *H. influenzae* have increased in some countries [6,7], although their global prevalence remains low [8]. Since biofilm-producing *H. influenzae* were recognized as a new issue in treatment, investigations of biofilm formation of antibiotics resistant strains such as BLNAR or β-lactamase-producing ABPC-resistant (BLPAR) became important. The aim of this study was to evaluate the biofilm formations of clinical isolates with different characteristics of antimicrobial susceptibility.

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2. Methods

2.1. Bacterial strains

All strains were stored at −70 °C. Strains were inoculated on chocolate agar plates and cultured at 37 °C overnight in 5% CO_2 for subculture. All strains were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, Mich, USA). Twenty-three type b strains were investigated using conventional polymerase chain reaction (PCR) with the capsule type b primers (b1–b2) that amplified capsule type-specific DNA fragments (480 bp). The sequences specific for Hib were 5′-GCGAAAGTGAACTCTTATCTCTC-3′ and 5′-GCTTACGCTTCTATCTCGGTGAA-3′ [9]. Region III–I primers (5′-GTGATTATTGATCTGCCCCCTAAAAGTTGGACAGGTTAG-3′ (15507–15545) and 5′-GGGGATAACATGACAACCG AAAATCCGGCAATACCGACG-3′ (20504–20542), GeneBank: AF5492 13) was designed for amplifying the partial *H. influenzae cap* loci including IS*1016*, *bexA*, *bexB*, and *bexC* genes, according to the nucleotide sequences described previously [10–12]. β-lactamase production was detected by means of a disc impregnated with nitrocefin (Becton Dickinson, Sparks, MD, USA).

2.2. Antimicrobial susceptibility test

MICs were determined by the agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute [13]. The susceptibilities of all the strains against the following 6 antibiotics were tested: Ampicillin (ABPC) (Meiji Seika Kaisha, Tokyo, Japan), Ampicillin/sulbactam (ABPC/SBT) (Pfizer Japan Inc, Tokyo), Cefotaxime (CTX) (Aventis Pharma, Tokyo), Cefotiam (CTM) (Takeda Chemical Industries, Osaka, Japan), Levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Tokyo) and Meropenem (MEPM) (Sumitomo Chemical Co., Tokyo).

2.3. PCR for identification of resistance genes

PCR for the identification of resistance genes was carried out for *H. influenzae* isolates using mixed primers (Wakunaga Pharmaceutical Co., Hiroshima, Japan), as described previously [14]. Briefly, P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae* (198 bp); TEM-I primers to amplify a part of the bla_{TEM-1} gene (458 bp); PBP3-S primers to identify an Asn526→Lys amino acid substitution in the *ftsI* gene (551 bp); and PBP3-BLN primers to identify an Asn526→Lys and Ser385→Thr amino acid substitution in the *ftsI* gene (465 bp). gBLNAS, gBLNAR and gBLPAR were determined followed the manufacturer's protocol.

2.4. Microtiter biofilm assay

Biofilm productions were investigated using a microtiter biofilm assay (MBA), as described previously [4]. Each strain was cultured for overnight in BHI and bacteria solution was diluted in 1:200 with fresh broth. 200 μl aliquots were inoculated into the wells of a 96 well micro-plate (Nalge Nunc International Co. Naperville, IL), and were incubated at 37C°, 5% CO_2 for 24 h. Before biofilm quantitation, bacteria growth was measured by using a micro-plate reader at OD_{490}. Twenty μl of freshly adjusted crystal violet (Fisher Scientific, Pittsburgh, PA) was added to each well and incubated at room temperature for 15 min. The plates were then washed vigorously with distilled water and dried. A volume of 230 μl of 95% ethanol was added to each well, and then the biofilm formation was measured at OD_{600}. All strains were tested in quadruplicate and the results were reported as average ± SD of three different experiments.

2.5. Visually observation of biofilms

2.5.1. SEM

The biofilm architectures were observed by scanning electron microscopy (SEM) [15]. In a typical experiment, coverslips were fixed in a 2% osmium 0.1 M Na cacodylate buffer for 1.5 h, dehydrated with ethanol washes, and dried with a critical point dryer to preserve the biofilm structure. The processed coverslips were then mounted on stubs using colloidal silver and were sputter coated with gold palladium.

2.5.2. Biofilm growth in a continuous-flow chamber

A continuous-flow chamber was used for biofilm formation as described previously [16]. 1 ml of BHI broth containing *H. influenzae* (1×10^8 cfu/ml) was inoculated into the chamber, and incubated for 1 h. BHI media diluted 1:10 with PBS (at a flow rate of 199.5 μl/min) was then added. Biofilms were allowed to form in a continuous-flow chamber for 2 days, and then observed by confocal laser scanning microscopy (CLSM), as previous study described [17]. In a brief, the flow chamber was carefully disconnected, biofilms formed on the surface of the glass coverslip of the chamber were stained with SYTO 9 and propidium iodide which were mixed at a 1:1 ratio (Molecular Probes, Eugene, Oreg), and incubated for 15 min at 37 °C. Biofilms were observed with a Bio-Rad MRC-1024 laser scanning confocal viewing system at a magnification of ×20. The resulting images were complied as cross sections of a z series.

3. Results

3.1. Characteristics of H. influenzae isolates

Twenty-six gBLNAS-NTHi strains were isolated from the nasopharynx (13 strains) and sputum (13 strains), 22 gBLNAR-NTHi were isolated from the nasopharynx (16 strains), middle ear (3 strains) and conjunctiva exudate of the eye (3 strains), 28 gBLPAR-NTHi strains were isolated from the nasopharynx (16 strains) and sputum (12 strains), and 23 Hib strains were isolated from the cerebrospinal fluid (20 strains) and nasopharynx (3 strains). Of the 23 Hib strains previously determined by slide agglutination assays, 18 (No. 5–19, 21–23) possessed the capsule type-specific DNA fragments (480 bp), and the partial Hib *cap* loci (5036 bp). While no targeted DNA fragments were detected in the remaining 5 strains (No. 1–4, 20), which suggested that mutations might have occurred in both investigated regions (Fig. 1A, B).

3.2. Antimicrobial susceptibility test

Table 1 showed the antimicrobial susceptibilities of all the strains against the 6 tested antibiotics against 26 gBLNAS, 22 gBLNAR, 28 gBLPAR-NTHi, and 23 Hib strains. The MICs of gBLNAR-NTH against ABPC, ABPC/SBT, CTX, CTM and MEPM were more resistant than those of gBLNAS, gBLPAR-NTHi and Hib. Whereas MICs against LVFX were low and similar among these 4 groups of *H. influenzae*.

3.3. Biofilm formation

The results of MBA were represented with the mean OD_{600} of gBLNAS, gBLNAR, gBLPAR-NTHi, and Hib strains as following 0.57 (range: 0.01–1.48), 0.50 (range: 0.13–1.19), 0.34 (range: 0.01–0.96), and 0.08 (range: 0–0.86), respectively. Variable abilities of biofilm production among gBLNAR, gBLNAS and gBLPAR-NTHi strains were detected (Fig. 2). Most Hib strains did not seem to produce biofilms, but 5 Hib strains showed significantly higher OD_{600} compared to those of the remaining Hib strains ($p = 0.0045$, Mann–Whitney's *U*

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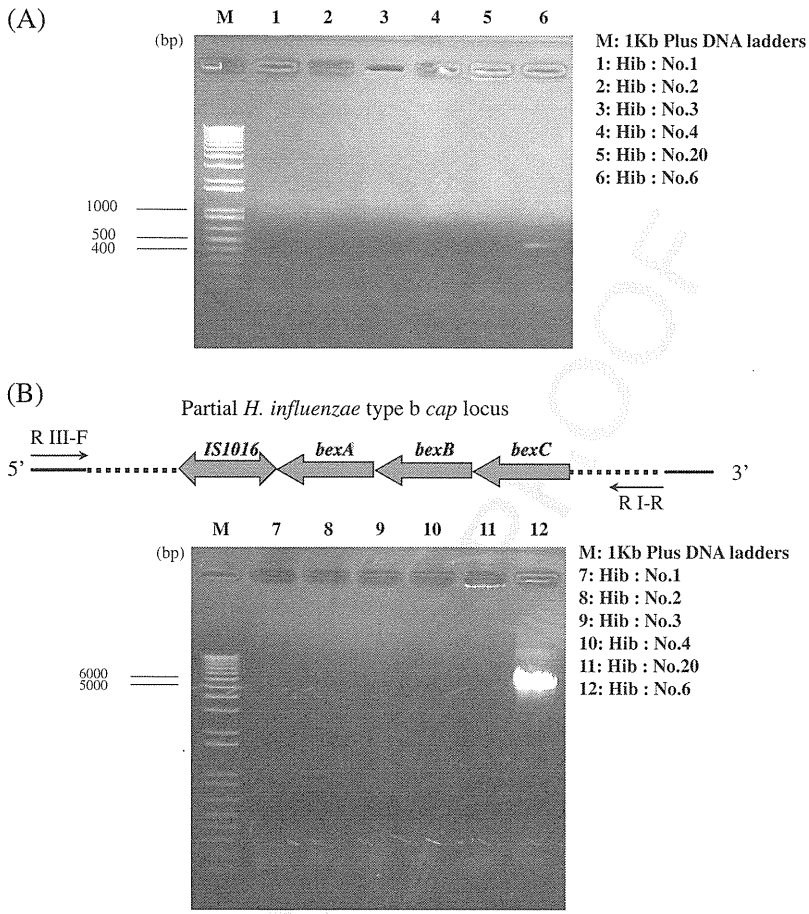

Fig. 1. A. Investigation of capsule type-specific DNA fragments. Capsule type b primers (b1–b2) were designed for confirmation of capsule type-specific DNA fragments (480 bp). B. Investigation of *H. influenzae* type b *cap* loci. Region II–I primers were designed to span a partial *H. influenzae* cap loci including IS*1016*, *bexA*, *bexB*, and *bexC* genes (5036 bp).

test). These 5 strains were confirmed with alternated Hib *cap* loci, 3 of which (No.1–3) were isolated from nasopharynx, and the remaining strains (No.4, No.20) were from cerebrospinal fluid.

NTHi strains were able to form mature biofilms with similar architectures, which were observed by SEM. On the other hand, the Hib strain (No. 6) did not produce biofilms with a weak attachment to the surfaces, but the No.3 strain formed a mature biofilm, which was confirmed with alternated type b *cap* loci (Fig. 3A). The same results have also been detected in a continuous-flow chamber with CLSM (Fig. 3B).

4. Discussion

Biofilm formation and the inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections [3]. Otitis media caused by NTHi constitutes one of such infections [3,5]. The emergence of drug-resistant NTHi such as the BLNAR strains has dramatically changed its clinical outcome in some countries [6,7], and not only antibiotics but also myringotomy is occasionally required as treatment. Although it has been reported that biofilm-producing *Pseudomonas aeruginosa* tend to be more resistant to antibiotics [18,19], there is still few information about the differences of biofilm formations between susceptible and resistant isolates such as BLNAR or BLPAR in *H. influenzae*. Our results demonstrate the capability of forming biofilms by resistant NTHi is similar to that by susceptible isolates overall, and which is different from *P. aeruginosa*. Furthermore, we found 9 (40.9%) and 14 (53.8%) strains in gBLNAR and gBLNAS-NTHi produced biofilms with OD_{600} value higher than 0.5, respectively, compared to 8 (28.6%) strains in gBLPAR-NTHi. These results suggested that β-lactamase might involve in *H. influenzae* biofilm formation. There is not any evidence to support this hypothesis in the present study, so we suggest future study should focus on this aspect.

The issue that whether Hib, the most common cause of bacterial meningitis in children, is capable to produce biofilm is still unclear. Previous studies demonstrated that biofilm matrix seemed to be regulated by CPS, protein components, and other extracellular molecules, and the rate at which bacteria attach to a surface is an important determinant of biofilm formation [20,21]. Our results demonstrated that most clinical Hib strains produced lesser amounts of biofilms compared to NTHi. Interestingly, we detected 5 Hib strains produced biofilms, and which were confirmed with alternated type b *cap* loci. As well known, all encapsulated *H. influenzae* strains contain functionally unique regions I, II, and III for the production of their respective polysaccharide capsules found within the *cap* loci, which is flanked by direct repeats of insertion element *IS1016* and is frequently amplified. Regions I (bexDCBA) and III are common to all six capsular types and contain

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Table 1
Antimicrobial susceptibilities of *H. influenzae* strains.

Antibiotics	Resistant class	MICs (μg/ml)		
		Range	50%	90%
Ampicillin	gBLNAS-NTHi	0.063–0.5	0.25	0.5
	gBLNAR-NTHi	1–8	2	8
	gBLPAR-NTHi	1–64	16	32
	Hib	0.125–64	1	8
Ampicillin/sulbactam	gBLNAS-NTHi	0.032–0.5	0.125	0.25
	gBLNAR-NTHi	0.5–2	1	1
	gBLPAR-NTHi	0.25–1	0.5	0.5
	Hib	0.032–2	0.25	0.25
Cefotaxime	gBLNAS-NTHi	0.008–0.063	0.016	0.032
	gBLNAR-NTHi	0.063–2	1	2
	gBLPAR-NTHi	0.016–0.063	0.016	0.063
	Hib	0.004–0.063	0.016	0.032
Cefotiam	gBLNAS-NTHi	0.5–1	1	1
	gBLNAR-NTHi	2–≧128	64	≧128
	gBLPAR-NTHi	0.5–2	1	2
	Hib	0.063–8	1	1
Levofloxacin	gBLNAS-NTHi	0.016–0.063	0.032	0.063
	gBLNAR-NTHi	0.008–0.063	0.032	0.063
	gBLPAR-NTHi	0.016–0.063	0.032	0.063
	Hib	0.008–0.063	0.032	0.063
Meropenem	gBLNAS-NTHi	0.016–0.125	0.063	0.063
	gBLNAR-NTHi	0.125–0.5	0.5	0.5
	gBLPAR-NTHi	0.063–0.25	0.063	0.125
	Hib	0.016–0.25	0.063	0.125

BLNAS: β-lactamase nonproducing-ampicillin susceptible *H. influenzae*; BLNAR: β-lactamase nonproducing-ampicillin resistant *H. influenzae*; BLPAR: β-lactamase producing-ampicillin resistant *H. influenzae*.

genes necessary for the processing and exportation of the capsular material, while region II is capsule type specific [11,12]. Thus we hypothesized that alternated type b *cap* loci might interrupt the expression of capsule and result in loosing the innate b capsule, and which might facilitate colonization and biofilm formation in these strains. Previous studies also reported that the frequency of spontaneous capsule loss in Hib is 0.1–0.3% [22], and capsule loss in Hib results in enhanced adherence to and invasion of human cells [23]. Although *H. influenzae* recovered from the respiratory tract generally showed non-encapsulated (serologically non-typeable), whereas isolates from systemic sites typically expressed encapsulated, especially a b capsule, some NTHi isolates from pharynx were proven to originate from an encapsulated ancestor [24].

Unfortunately, we could not investigate the capsule variation at a protein level, as well as the relatedness between biofilm formation and capsule, future work should focus on these aspects. It has also been reported that the expression of capsule polysaccharide (CPS) in *Vibrio vulnificus* inhibits biofilm formation, and impaired pneumococcal CPS may increase biofilm formation and involve in the inhibition of the virulence possibly due to influencing the gene expression in *Streptococcus pneumoniae* [20,25]. The mechanism for why the loss of b capsule in Hib leads to enhance biofilm formation seemed to be complicate. Pili has been reported as an important structure for adherence [4], and biofilm formations [26,27] in *H. influenzae*. Since most Hib strains seemed not to produce biofilms, we thus speculate that a capsule might shield the function of the pili in the prior attachment was responsible for impaired biofilm formation.

On the other hand, genes involved in capsule expression are major virulence factors of *H. influenzae* [11,28]. It has been recently reported that a *H. influenzae* type a strain isolated from cerebrospinal fluid of a child with meningitis and a non-encapsulated (serologically non-typeable) *H. influenzae* strain simultaneously obtained from blood of the same patient showed the similar genetic structure for the cap region [10]. These data suggested that there is a possibility that Hib strains even lose their capsule and colonize in the nasopharynx, which may still have sufficient virulence to cause an invasive disease in human, and which is a high concern for clinicians.

Our data indicate that *H. influenzae* may produce mature biofilms without regard to their resistant status. Hib strains with alternated type b *cap* loci produced biofilms might be due to interruption of the b capsule expression. Therefore, future research conducting the relatedness of the loss of capsule, biofilm formation and virulent factors should be necessary.

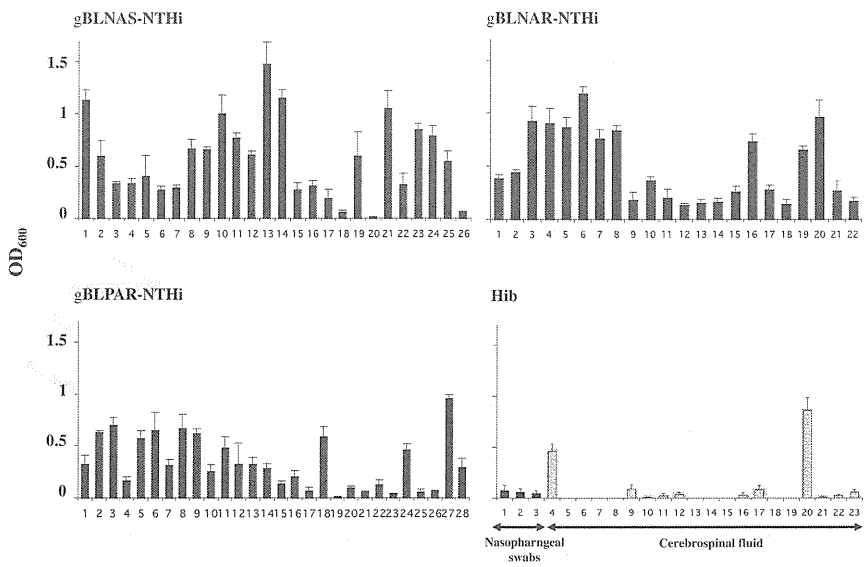

Fig. 2. Biofilm formation in each resistant class of *H. influenzae* strains. BLNAS: β-lactamase nonproducing-ampicillin susceptible *H. influenzae*; BLNAR: β-lactamase nonproducing-ampicillin resistant *H. influenzae*; BLPAR: β-lactamase producing-ampicillin resistant *H. influenzae*. All strains were tested in quadruplicate and the results were reported as average ± SD of three different experiments.

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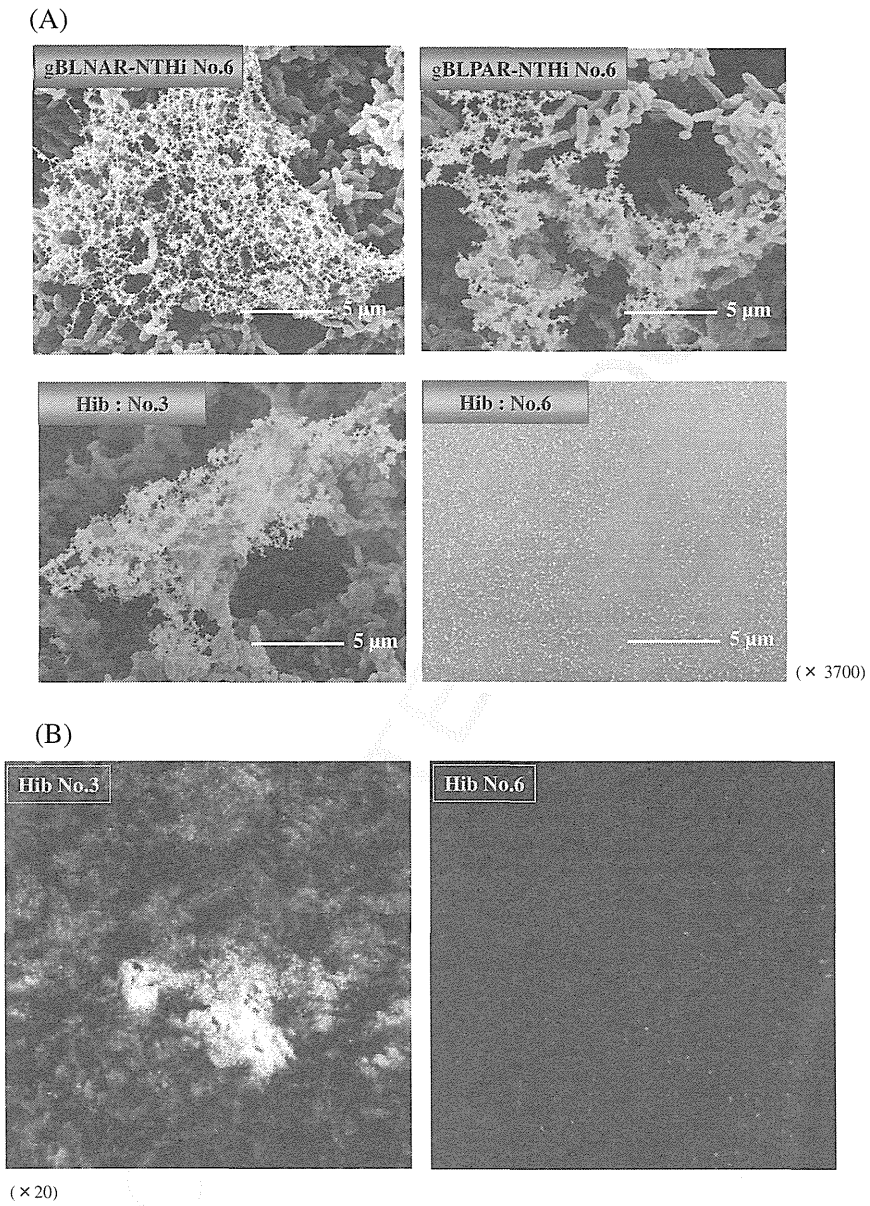

Fig. 3. A. Biofilm architectures observed by SEM. SEM showed that gBLNAR-NTHi (No. 6), gBLPAR-NTHi (No. 6), and Hib strain (No. 3) clearly produced mature biofilms, whereas Hib strain (No. 6) did not produce a biofilm. B. Biofilm architectures in a continuous-flow chamber observed by CLSM. Biofilms grown in the chamber were observed with a Bio-Rad MRC-1024 laser scanning confocal viewing system at a magnification of ×20. The resulting images were complied as cross sections of a z series. CLSM showed the Hib strain (No. 3) was able to form a biofilm, but the Hib strain (No. 6) failed to produce a biofilm.

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The authors declare that they have no conflict of interest.

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Performance of a rapid human metapneumovirus antigen test during an outbreak in a long-term care facility

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SUMMARY

Using a newly developed rapid test, an outbreak of human metapneumovirus (HMPV) infection in a long-term care facility was detected within only 2 days after the onset of symptoms in a putative index case. The outbreak was almost under control within 8 days mainly by zoning patients, with the exception of two cases of HMPV that were diagnosed 16 and 17 days after the onset of the outbreak. According to an immunological diagnosis as well as the rapid test, it was eventually proven that 18 patients had HMPV infections. We suspected that even asymptomatic residents, who had not been completely separated from the facility population, were a source of infection. That suggested that all asymptomatic residents should be tested and that the separation of the infected patients should be absolute, if an outbreak of HMPV infection is suspected in such a facility.

Key words: Human metapneumovirus, long-term care facility, rapid HMPV antigen test.

INTRODUCTION

Human metapneumovirus (HMPV) is an important pathogen of the lower respiratory tract that most often affects hospitalized children, immunocompromised patients [1] and elderly occupants of long-term care facilities [2, 3]. Due to the close proximity of living quarters and the reduced levels of personal hygiene and movement of the residents, outbreaks of fatal influenza or norovirus infections have been reported in such facilities [4, 5]. A rapid test has become a very useful tool in preventing the spread of infections because it results in rapid identification and isolation of the index case. A rapid HMPV antigen test has not been available until recently and the sensitivity and specificity of the assay have been described [6]. The present study focused on evaluating the performance of this new HMPV rapid test during a recent outbreak of HMPV infection in a long-term care facility.

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METHODS

Nasopharyngeal swab specimens were tested using both the Check hMPV assay (Meiji Seika Pharma Co. Ltd, Japan) and a nested RT–PCR [7]. The amplicon fragment (357 bp) was extracted using

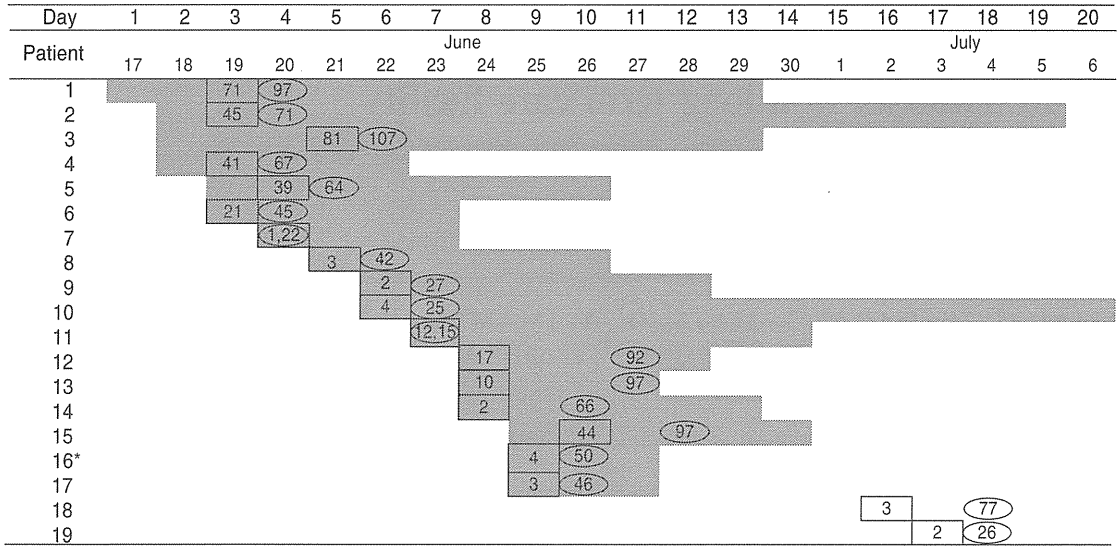

Fig. 1. The time-course of the outbreak of human metapneumovirus (HMPV) infection. The grey shading indicates the duration of fever (body temperature >37·5 °C); rectangles indicate the day of diagnosis by the rapid test (numbers within rectangles represent hours after onset of symptoms); ellipses indicate the day of collection of the acute serum and nasopharyngeal swab (for HMPV RNA extraction) (numbers within ellipses represent hours after the onset of symptoms). * Patient 16 was accommodated in an intensive respiratory care unit on 28 June.

GeneCleanII (MP Biomedicals, USA). The sequence was determined using a primer specific for the HMPV F gene (5′-CATGCCGACCTCTGCAGGAC-3′, 5′-ATGTTGCAYTCYYTTGATTG-3′) (FASMAC, Kanagawa, Japan). This rapid test was previously known as the SAS hMPV test [6]. An indirect fluorescent antibody test for measuring IgM and IgG antibodies was performed to confirm the HMPV infection as described previously [2, 8]. The serological test was performed once on the serum of all patients. In addition to the detection of viral antigens and nucleic acids, a fourfold increase in serum HMPV IgG titre 2 weeks after onset of symptoms provided evidence of positive immune responses to HMPV. For virus isolation, nasopharyngeal swab samples were suspended in Eagle's minimum essential medium (MEM), and the suspension was inoculated onto LLC-MK2 and Vero E6 cells. The cells were cultured in MEM containing trypsin (1 μg/ml). Isolation of HMPV was confirmed via RT–PCR and the rapid test. The present study (number 09040) was approved by the Ethics Committee of Kurume University.

RESULTS

A total of 18 patients were affected with HMPV over a 20-day period during June–July 2011, in a long-term care facility in Kurume, Japan (Fig. 1). One of 45 residents (case 6) on the first floor and 17 of 41 residents on the second floor were affected in one of several building units in this facility. All residents of each floor were accommodated in one open space. Most patients had a fever lasting 3–6 days. There were 1–3 new patients diagnosed daily during the first 8 days. Using the rapid test, we detected the outbreak in only 2 days after the onset of symptoms in the putative index case (case 1). Infection control measures were instituted to prevent the further spread of infection, which included zoning patients and hand washing. The zone where patients resided was surrounded by a long vinyl curtain in a corner of the ward. Additional patients continued to be diagnosed over the 4- to 5-day HMPV infection incubation period [9]. However, the outbreak was almost under control within only 8 days. Two patients were diagnosed 16 and 17 days after the onset of the outbreak. Case 16 was admitted to the intensive-care unit of another hospital with severe respiratory failure. The attack rates on the first and second floors were 2·2% and 44%, respectively. The total attack rate was 19·8%, which was smaller than that in previous reports [2, 10]. There was no record concerning HMPV infection in staff members.

According to an immunological diagnosis, as well as to the rapid test, it was eventually proven that all symptomatic patients, except for case 11, had been

Table 1. *Clinical features of symptomatic patients during a human metapneumovirus (HMPV) outbreak*

Patient no.	Age (yr)	Sex	WBC (/mm^3)	CRP (mg/dl)	Rapid test*	RT–PCR†	Virus‡	IgM titre			IgG titre			
								Acute	2 wk	1 yr§	Acute	2 wk	Ratio	1 yr§
1	32	M	7000	5	+	+	–	20	80	<10	160	2560	16	320
2	28	M	4400	3	+	+	–	<10	10	<10	80	2560	32	80
3	6	F	3700	2	+	+	–	160	320	20	320	10240	32	640
4	17	M	7000	2	+	+	–	40	40	<10	80	10240	128	160
5	17	M	9400	0	+	+	–	1280	160	40	40	5120	128	40
6	42	F	7600	6	–	–	–	80	40	80	10	320	32	640
7	13	M	5200	0	–	–	–	640	80	10	1280	5120	4	320
8	41	M	4600	2	+	+	–	160	320	<10	640	5120	8	160
9	21	M	7400	12	+	+	+	320	160	<10	640	2560	4	160
10	42	F	7700	2	+	+	–	40	160	<10	1280	10240	8	320
11	40	M	5300	2	–	–	–	320	<10	<10	1280	2560	2	80
12	27	M	12000	1	+	+	–	10	80	80	1280	2560	2	160
13	36	F	6800	4	+	+	–	80	80	<10	40	5120	128	80
14	43	M	5400	6	+	+	–	40	80	<10	320	5120	16	160
15	4	F	8400	4	+	+	+	320	160	<10	640	5120	8	80
16	26	M	9000	5	+	–	–	160	320	<10	2560	5120	2	80
17	41	F	6200	1	+	+	–	320	320	<10	40	1280	32	160
18	3	F	8200	0	+	+	+	640	1280	<10	160	1280	8	80
19	10 mo.	F	11100	<0·2	+	+	–	320	160	40	20	160	8	160

WBC, White blood cells; CRP, C-reactive protein.
* Check hMPV assay (Meiji Seika Pharma Co. Ltd, Japan).
† Genbank accession numbers of the amplicon (F gene) are JX966477–JX966485.
‡ Virus isolation usingLLC-MK2 cells.
§ IgM and IgG titres were evaluated 1-year post-outbreak of HMPV.

infected with HMPV (Table 1). Of these, 16 (84%) patients were positive for HMPV according to the rapid test result. These results were confirmed using a nested RT–PCR, with the exception of case 16. HMPV was isolated in the samples from cases 9, 15 and 18. Cytopathic effects of LLC-MK2 cells were seen and rounded and fused cells were clearly observed. The sequences of the amplicon, which codes for a part of the HMPV F protein, were completely identical in all sequences obtained (GeneBank accession numbers JX966477–JX966485), which suggested that the outbreak of HMPV infection would have originated from an index case.

Figure 1 shows the duration of fever and the time lag between the onset of symptoms and the diagnosis (rapid test, RT–PCR, immunological test). The time lag of the rapid test in nine cases was around 1–4 h. Of these nine cases, only case 7 was negative by the rapid test. The time lag for the remaining cases was between 10 and 81 h. Of these cases, only two were found to be negative by the rapid test. The time lag was not related to the detection rate. Based on the RT–PCR results, the negative results were evenly detected in several cases. The time lag between the onset of symptoms and the collection of the nasopharyngeal swab for RNA extraction varied from short to long.

DISCUSSION

In this study, we were able to detect an outbreak of HMPV infection in a long-term care facility at an early phase using a new rapid test. Early detection of HMPV enabled us to prevent widespread infection at the facility and the outbreak had almost ceased within 8 days. We were also able to keep the affected number of patients to within 17 out of a total of 41 residents in a ward on the second floor and to 1 out of 45 in another ward on the first floor.

Influenza outbreaks have been reported in a long-term care facility and fatal cases are common. Many patients in this report had a high fever for at least 3–7 days (Fig. 1). This period seemed to be longer than that seen for influenza infections. However, fatal cases of HMPV infection are relatively rare [2, 11]. Early detection of the HMPV outbreak increased the prospects for a good outcome.

In total, 16 (89%) of the 18 HMPV patients were found to be positive using the rapid test. The rapid

test and RT–PCR results were in accord except for case 16. The time lag between the onset of symptoms and the collection of a nasopharyngeal swab for RT–PCR was 50 h, but the time lag between the onset of symptoms and the rapid test was only 4 h. That suggested that the amount of HMPV might have decreased by the time the nasopharyngeal samples were collected. With respect to the rapid test-positive results for cases 12 and 16, no significant increase in serum IgG titre was detected 2 weeks after the onset of symptoms, because the titre of IgG in acute serum had already increased. This might suggest that cases 12 and 16 were asymptomatically infected with HMPV at a much earlier time, although the time lag between the onset of symptoms and the collection of serum samples was relatively early (within 3–4 days). The role of asymptomatic patients in comparatively large HMPV outbreaks has been reported previously [12].

False-negative rapid test results were noted for cases 6 and 7. These patients were also immediately isolated in the same separated area as the rapid test-positive patients because they showed similar clinical symptoms. It is important to note that cases 6 and 7 eventually tested positive for HMPV according to the serological test. This result suggested that the isolation of all symptomatic patients, including patients with false-negative results, was an appropriate control measure to prevent the spread of infection.

We concluded that the rapid HMPV test had high sensitivity, making it very useful for the early detection of an outbreak of HMPV infection in a long-term care facility. Our study also suggested that even asymptomatic patients might be a source of infection. Therefore, we propose rapid diagnosis testing for all asymptomatic residents as well as for symptomatic patients. Testing the asymptomatic residents might be an important measure in preventing the emergence of secondary-infected patients in such a long-term care facility.

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DECLARATION OF INTEREST

None.

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